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Chicken growth hormone (cGH), a polypeptide hormone synthesized in and secreted by the pituitary gland, is involved in a wide variety of physiological functions such as growth, body composition, egg production, aging, and reproduction. Chicken growth hormone polymorphisms have been reported to be associated with certain phenotypes. Our objective is to investigate the GH gene polymorphism in selected strains of native Chinese chickens. Yellow Wai Chow GH gene was characterized by sequencing and was found to have one silent substitution, 31 insertions, and other substitutions spread among the introns. In addition, a novel *MspI* site has been identified and characterized in the first intron. Allele frequencies of the intron 1 polymorphism were characterized among 28 populations of native Chinese chickens. Thus, polymorphism of the cGH gene may be useful in phylogenetic analysis, as well as in the design of breeding programs. [Exp Biol Med Vol. 226(5):458–462, 2001]

Key words: growth hormone; polymorphism; chicken; allele frequency

Growth hormone (GH) affects a wide variety of physiological parameters such as growth performance, carcass composition, and milk production (1–3). The genomic structure of the GH gene has been studied in different animals, including rat (4), bovine (5), sheep (6), pig (7), human (8–10), goat (11), chicken (12), and mice (13). These animals share a similar gene structure containing five exons and four introns. The chicken GH (cGH) gene is similar to mammalian GH genes. However, the introns of cGH were significantly larger. Studies on meat-type chickens using restriction fragment length polymorphism (RFLP) shows that the GH gene is highly polymorphic in the intron region and alleles are identified for the selection of abdominal fat (14). Kuhnlein (15) analyzed 12 noninbred strains of White Leghorn chicken by PCR-RFLP at three *Msp*I sites (PM1, PM2, and PM3) and one *Sac*I site (PS1). They suggested that these alleles, located within the introns, were selected either for an array of egg production traits, resistance to Marek's disease, or resistance to avian leukosis (15).

GH gene polymorphisms have also been observed in other poultry and animals. A recent study on dairy cattle demonstrated that an *Msp*I polymorphism at intron 3 of bovine GH (bGH) gene was linked to the content of milk protein (16). PCR-RFLP studies on artificial insemination (AI) bull also showed that GH gene polymorphism is associated with the reproduction performance of AI bull (17).

In the present study the cGH gene of native Chinese chicken, Yellow Wai Chow (YWC) strain, was sequenced and compared with that of a White Leghorn strain. Allele frequency of PM3 was also determined by PCR-RFLP in 28 strains of chickens derived from four different genetic background bases (Chinese native chicken, hybrids of Chinese native breeds, and imported broilers, broilers, and layers). The aim of the present study was to use PCR-RFLP analysis to investigate GH gene polymorphisms in the selected Chinese strains of chickens.

Materials and Methods

Chicken Populations. Genomic DNA samples were collected from four chicken populations: Chinese native chicken, hybrids from native Chinese breeds and imported broilers, broilers, and layers. Native Shek Kai (n = 15), YWC strain (n = 8), Huiyang Bearded (n = 36), Xinghua (n = 35), Taihe Silkies (n = 32), Gushiu (n = 24), Beijing Fatty (n = 24), and Wenchung (n = 24), Qingyuan are Chinese native breeds. Among them, the Qingyuan breed was further divided into two groups: commercial chicken (Qingyuan 1, n = 20) and breeder (Qingyuan 2, n = 30). Taihe Silkies is a silkies breed. Huiyang Bearded has a beard and Beijing Fatty has feathered shanks and feathered comb. YWC strain had been inbred from Huiyang for over 20 years. Shek Kai Pure (n = 20), Shek Kai (n = 20), Shek Kai B (n = 20), Shek Kai AFD (n = 20), Beijing Shek Kai (n = 20), Shek Kai

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Hybrid ($n = 17$), Partridge Line 1 ($n = 13$), Partridge Line 2 ($n = 72$), Yellow Line 1 ($n = 20$), Yellow Line 2 ($n = 35$), Xinxing Yellow ($n = 62$), and NK272 ($n = 30$) were collected from the hybrids of Chinese native chickens and broilers, but bred at different times. Kabir 1 ($n = 19$), Kabir 2 ($n = 22$), and Avian Parental ($n = 22$) are broiler strains. Hybrid Line 1 ($n = 30$) and Hybrid line 2 ($n = 91$) are pure lines of Leghorn chickens. We have also included Black Silkies ($n = 30$) in this study.

Sequencing of cGH Gene of YWC Strain. The genomic sequence reported by Tanaka *et al.* (12) was used to design a pair of primers for the amplification of the cGH gene. A PCR product (3374 bp) was obtained and cloned into pMOSBlue vector. Plasmid DNA was extracted and the cGH gene was sequenced. A total of seven pairs of primers were designed for the DNA sequencing and sequencing was carried out on sequence analyzer ABI PRISM 310.

PCR-RFLP of Intron 1. Intron 1 was amplified by PCR and the two primers were 5′-ATCCCCAGGCAAACATCCTC-3′ (PM3 forward) and 5′-CCTCGACATCCAGCTCACAT-3′ (PM3 reverse). For each PCR reaction, 100 ng of genomic DNA sample was added to 50 μl of reaction mix containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 50 pmole each primer, 1.5 Units *Taq* polymerase, and 5 μl of 10× PCR buffer (Life Technologies, Grand Island, NY). Both primers were described previously by Tanaka *et al.* (12) and gave a predicted product of 776 bp. The PCR conditions used comprised an initial denaturation step of 95°C for 4 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 2 min, and an extension step of 72°C for 90 sec.

PCR product (8–12 μl) was then digested overnight at 37°C in a reaction mix that contained 1 μl *Msp*I (about 5 U), 1.5 μl of Reaction 1 buffer (Life Technologies), and water to give a final volume of 15 μl. The digested DNA was then analyzed on a 1.5% agarose gel, run at 100V for 2 hr. The DNA fragments were stained with ethidium bromide and photographed using a UV transilluminator to visualize the products. Allele frequency of RFLP in each population was then calculated.

Results

Sequencing of cGH Gene of YWC Strain. A 3374 bp (545–3716 bp) fragment of the YWC chicken GH gene was sequenced. Table I shows substitutions and insertions observed in both the intron and exon regions of YWC strain cGH gene when compared with that of a White Leghorn strain. A high frequency of substitution and insertion was observed in intron regions, especially in intron 1 in which a total of three insertions and 12 substitutions were found. On the other hand, there was only one substitution found in exon 4 at 2338 bp and it was a silent mutation (i.e., CTC to CTG) in which both encode for Leu. The numbers of changes for the introns when normalized to a 1-kb length are 21, 5, 3, and 7 for the YWC chicken, respectively. The first intron showed the highest number of changes while the third intron had the lowest.

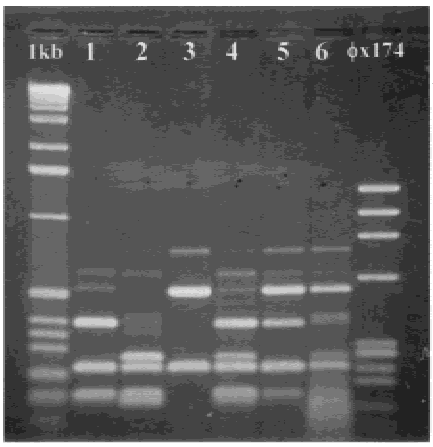
PCR-RFLP of Intron 1 in Chinese Native Chicken cGH Gene. A PCR product of the expected size (776 bp) was obtained using PM3 primers [4]; it was then digested with *Msp*I. Six different profiles were found in intron 1 (Fig. 1) and they were created by the combination of a polymorphic *Msp*I-cut site (PM3), nonpolymorphic *Msp*I-cut site, and a new *Msp*I-cut site, which was present in the 414-bp fragment. Profile 1 was determined by allele I in which two restriction MspI sites were cut, but not the new MspI site, and fragments of 414, 237, and 125 bp, respectively, were produced. Profile 2 was determined by allele II in which all of the three MspI sites were being cut, producing four fragments of 267, 237, 147, and 125 bp, respectively. Profile 3 was determined by allele III in which there was only one restriction MspI site cut (i.e., MspI site) and the two other MspI sites were not digested (PM3 and new MspI). Two fragments of 539 and 237 bp were produced. Profiles 4 to 6 were heterzygotes consisting of alleles I, II, and III.

Direct sequence of the PCR product was analyzed. The results showed that the new *Msp*I-cut site was formed by a substitution of a T residue "ccTgg" with a G residue "ccGgg" + 133 bp of the *Pst*I fragment reported by Mou *et al.* (18). It was also found that this new *Msp*I-cut site was digested only when PM3 site was cut.

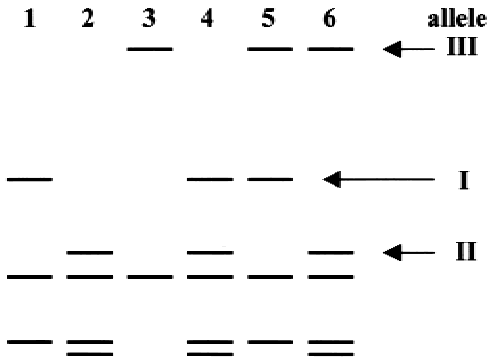
Allele Frequencies of 28 Populations of Native Chinese Chickens and Other Breeds. Allele frequencies of 28 populations of native Chinese chickens with intron 1 polymorphisms are shown in Table II. A similar pattern of allele frequency was observed between native

Table I. Comparison of YWC Strain cGH Gene Sequence and White Leghorn Strain cGH Gene Sequence by Tanaka *et al.* (12), Modified by Mou *et al.* (18) as References

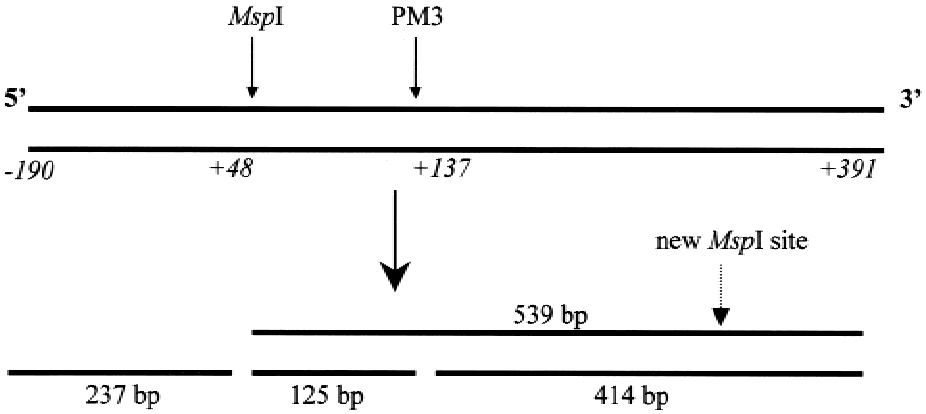
Exon				Intron			
Exon number	Size	Insertions	Substitutions	Intron number	Size	Insertions	Substitutions
1	66	Nil	Nil	1	915	4	17
2	161	Nil	Nil	2	442	Nil	2
3	117	Nil	Nil	3	301	Nil	1
4	162	Nil	1	4	1068	2	5
5	198	Nil	Nil				
Subtotal	704	Nil	1	Subtotal	2726	6	25



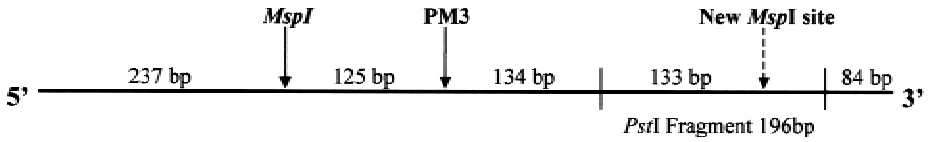
(A) Profiles of intron 1



(B) RFLP patterns of intron 1



(C) Restriction map of Intron 1



(D) Location of new *Msp*I cut site.

Figure 1. RFLP and mapping of the *Msp*I polymorphism in the first intron of the cGH gene. (A) Profiles of intron 1. (B) RFLP patterns of intron 1. (C) Restriction map of intron 1. (D) Location of new *Msp*I restriction site.

Chinese chickens and broilers (i.e., Kabir 1, 2, and Avian Parental). However, distinctive differences in allele frequencies were observed between native Chinese chicken and a layer strain (i.e., Hy-Line). A high frequency of allele III (0.950) and a low frequency of allele II (0.00) were observed in the Leghorn Hy-Line strain, which has been selected for egg laying performance. Based on the allele frequencies among different populations, either the absence of allele II or high allele frequency of allele III could be linked to laying performance of the chicken.

Discussion

In recent years, DNA polymorphisms (mainly in introns) have been widely studied in the GH gene of various animals. A polymorphism, which was located in intron 3 of the bGH gene, was found to be associated with the selection for milk fat and protein contents (16, 19, 20). GH gene polymorphism identified in AI bulls affects sperm production and reproductive performances of bulls (17). As reported by Kuhnlein *et al*. (15), RFLPs were identified in

Table II. Allele Frequencies of Intron 1 Polymorphisms in Different Populations

Allele	I	II	III	Allele	I	II	III
Native Chinese chickens (N)*				Hybrids (N)*			
Native Shek Kai (15)	0.600	0.200	0.200	Shek Kai Pure (20)	0.725	0.175	0.100
YWC strain (8)	0.375	0.125	0.500	Shek Kai (20)	0.675	0.100	0.225
Huiyang Bearded (36)	0.680	0.080	0.240	Shek Kai B (20)	0.425	0.350	0.225
Xinghua (35)	0.630	0.160	0.210	Shek Kai AFD (20)	0.375	0.250	0.375
Taihe Silkies (32)	0.670	0.330	0	Beijing Shek Kai (20)	0.470	0.290	0.240
Gushiu (24)	0.750	0.125	0.125	Shek Kai Hybrid (17)	0.620	0.270	0.110
Beijing Fatty (24)	0.690	0.080	0.230	Partridge Line 1 (13)	0.475	0.325	0.200
Wenchung (24)	0.600	0.130	0.270	Partridge Line 2 (72)	0.576	0.271	0.153
Qingyuan 1 (20)	0.620	0.180	0.200	Yellow Line 1 (20)	0.600	0.150	0.250
Qingyuan 2 (20)	0.520	0.320	0.160	Yellow Line 2 (35)	0.657	0.086	0.257
				Xinxing Yellow (62)	0.613	0.081	0.306
				NK272 (30)	0.484	0.067	0.449
Broilers (N)*				Others (N)*			
Kabir 1 (19)	0.475	0.125	0.400	Hy-Line 1 (30)	0.050	0	0.950
Kabir 2 (22)	0.360	0.290	0.340	Hy-Line 2 (91)	0.132	0	0.868
Avian Parental (22)	0.630	0.070	0.300	Black Silkies (30)	0.470	0.150	0.380

(N)*, Number of chickens.

cGH gene of a White Leghorn strain at three *Msp*I sites (PM1, PM2, and PM3) and at a *Sac*I site (PS1). These sites were located at intron 1 (PM3), intron 3 (PM2), and intron 4 (PM1, PS1) respectively. Table II shows that a higher number of substitutions and insertions were observed in the intron regions (31/32 = ~97%) than in exon one (1/32 = ~3%), as found in the cGH gene of YWC strain when compared with that of the White Leghorn strain as reported by Tanaka *et al.* (12). The number of substitutions and insertions, as compared with YWC, was found to be the highest in intron 1 (21/31 = 67%). However, intron 4, which is similar to intron 1 in size, had only 22% of the substitutions and insertions (7/31). When the number of changes among all four introns is normalized to 1 kb, intron 1 is shown to have the highest number of changes, 21, when compared with intron 4 (seven changes). Other changes were found in intron 2 (five changes) and 3 (three changes). A glucocorticoid regulatory element (GRE) has been located in the first intron of the human GH (hGH) gene and could be involved in the transcriptional control of the hGH gene expression (21, 22). It is possible that the high number of substitutions and insertions observed in the present study could be involved in the expression of the cGH gene. The high number of changes in sequence observed in the present study could be due to selection pressure. A silent mutation was observed in exon 4 of YWC strain as compared with White Leghorn strain in which the codon at 2338 bp, CTC, was modified to CTG, although both of these codons encode the same amino acid, leucine. Although the effect of this substitution remains unknown, a substitution was observed in bGH in the amino acid position 127 (leucine to valine) that could affect the characteristics of GH secretion (23).

In determining the RFLP in intron 1, four different types of Chinese native chicken were studied. Six different profiles were found when intron 1 PCR products were digested with *Msp*I, as shown in Fig. 1, A and B. Profiles 1 through 3 were that of the homozygote genotype. Profiles 1 and 3 are formed by the two *Msp*I sites reported by Mou *et al*. (18) in which one of them was polymorphic (PM3), while another was nonpolymorphic (see Fig. 1C). A novel polymorphic MspI site (profile 2) was located at the position of +133 bp of the *Pst*I fragment reported by Mou *et al.* (18). This new *Msp*I site occurs only with PM3, demonstrating an allelic association or linkage disequilibrium.

Table II shows the allele frequencies of different types of native Chinese chickens. Allele I, having the highest allele frequency (average = 0.54), was identified as the dominant allele in the RFLP among all types of chicken (except Hy-Line), while Allele II had the lowest allele frequency. It may be suggested that allele II was created by a new *Msp*I site by a mutational event. Therefore, its appearance would be the lowest (average = 0.18) among three types of alleles. It should be noted that there was a significant difference between the allele frequency of Leghorn Hy-Line strain and that of other native Chinese chickens. A high frequency of allele III (frequency = 0.95) and low frequency of allele II (frequency = 0.00) was observed in Hy-Line chickens. Since the Leghorn Hy-Line strain has been selected for egg laying performance, either the absence of allele II or a high allele frequency of allele III could be linked to laying performance. The formation of allele II was closely associated with the novel *Msp*I site and was identified only in native Chinese chicken. Whether the absence of this specific allele is important for the selection of laying performance remains to be clarified.

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